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Enhancement of immune response to naked DNA vaccine by immunization with transfected dendritic cells

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Abstract: Immunization with plasmid DNA encoding various proteins promises to be a valuable vaccine approach especially if its immunogenicity could be optimized. In this study we show that the intramuscular delivery in dendritic cells (DC) of naked plasmid DNA encoding two proteins of herpes simplex virus (HSV) leads to the induction of significantly enhanced levels of resistance to viral challenge. Whereas DC transfected in vitro with DNA induced enhanced immunity, similarly transfected macrophage (Mϕ) populations lacked immunogenicity even though plasmid expression occurred in vitro. The enhanced immunity induced by DC-delivered DNA appeared to be associated mainly with an increased Th1 $CD4^+$ T cell response. Our results add evidence that DC are the essential antigen-presenting cell types involved in immune responses to intramuscularly administered DNA vaccines. *J. Leukoc. Biol.* 61: 125–132; 1997.

Key Words: herpes simplex virus · macrophages · anti-herpes · cytokines · lymphocytes · nucleic acid vaccine · plasmid DNA

INTRODUCTION

Reports in 1992–1993 that naked DNA (nDNA) could be used to induce immunity ushered in the era of nucleic acid vaccines [1–3]. The primary observations on influenza virus have been extended to several other agents, which include viruses, bacteria, and certain parasites [reviewed in refs. 4–6]. Many hope that nDNA vaccines will find a place in the market but certain deficiencies must be overcome before the nDNA approach supplants any currently effective vaccine. Typical of one problem is the result we have obtained using nDNA vaccines against herpes simplex virus (HSV) infection in mice. Protection was achieved with plasmid DNA encoding at least two different proteins, a structural major glycoprotein as well as a nonstructural immediate early protein [7–8]. However, in comparison with immunization with HSV or with recombinant vaccinia viruses expressing HSV proteins, levels of protection were inferior [9]. A challenge facing nDNA vaccinology is to find means of enhancing immunogenicity.

Although the mechanisms by which nDNA vaccines given intramuscularly achieve immunogenicity are still unresolved, it is most likely that the plasmid expression by invading professional antigen-presenting cells (APC) is responsible for immune induction [10]. It is conceivable that dendritic cells (DC), with their compendium of properties important for immune activation, are crucially involved (11). Indeed, an ever-increasing collection of proteins, peptides, or even mRNA incorporated into DC in vitro enhances the level of immune induction subsequently achieved in vivo [12–18]. An early observation came from our own group showing that incorporation of peptides into DC enhanced immune responses against HSV [19]. We have also shown that transfection of DC in vitro with nDNA encoding HSV proteins markedly enhances the subsequent primary immune response achieved in vitro against HSV [20]. In this study, we present the novel observation that immunization with DC transfected with nDNA leads to a significant enhancement in protective immunity against an infectious agent. This effect fails to occur with macrophages (Mϕ) similarly transfected with nDNA. Our results are discussed in terms of the likely mechanisms of immunity responsible for improved protection and the relevance of using nature's adjuvant [11] in practical vaccinology.

MATERIALS AND METHODS

Mice

Female BALB/c mice, 3- to 4-weeks old, purchased from Harlan Sprague-Dawley, Indianapolis, IN were used in this study. The investigators adhered to the guidelines of the Committee on the Care of Laboratory Animal Resources, Commission on Life Sciences, National Research Coun-

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Abbreviations: gB, glycoprotein B; ICP-27, infectious cell polypeptide 27; PFU, plaque-forming unit; DC, dendritic cell; Mϕ, macrophage; HSV, herpes simplex virus; nDNA, naked plasmid DNA; DC-DNA, dendritic cells transfected with plasmid DNA encoding HSV protein; APC, antigen-presenting cells; FCS, fetal calf serum; PBS, phosphate-buffered saline; DOTAP, *N*-[1-(2,3-dideoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate; PCS, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RIPA, radioimmunoprecipitation assay; IFN-γ, interferon-γ; IL, interleukin; DTH, delayed-type hypersensitivity; CTL, cytotoxic T cell assay; GM-CSF, granulocyte-macrophage colony-stimulating factor.

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Received September 3, 1996; revised November 13, 1996; accepted November 14, 1996.

cil and the facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.

Virus

HSV-1 strain 17 and KOS were grown on vero cell monolayers (no. CCL81, American Type Culture Collection, Rockville, MD) and stored at −80°C until use.

DNA preparation

Plasmid (pc-DNAI) DNA expressing glycoprotein B (gB) or infectious cell polypeptide 27 (ICP-27) of HSV-1 was constructed as described [7, 8]. Briefly, a cDNA clone of HSV-1 (KOS) gB (kindly provided by Dr. Martin Muggeride, Wistar Institute), a 3.7-kb HindIII-BamHI fragment was subcloned into vector pcDNAI (Invitrogen, San Diego, CA) allowing expression of gB by a cytomegalovirus promoter. This clone (pc gB) was transformed into *Escherichia coli* MC1061 strain (Invitrogen) and stored at −70°C in a 15% saline/0.1% glycerol solution. Bacteria were grown in Lennox L Broth (Life Technologies, Paisley, Scotland, catalog no. 12780-052) and plasmids were isolated using Megaprep plasmid isolation columns (Promega, Madison, WI). Purity and concentration of DNA were determined as described previously [20]. A similar procedure was used for the construction of ICP-27 DNA. A 2.4-kb ICP-27 cDNA was cloned into M13mp18 and was subcloned into HindIII-XbaI sites.

DC isolation

Syngeneic DCs were isolated as per the procedure mentioned elsewhere [21]. Briefly, BALB/c splenocytes were obtained and the cell concentration was adjusted to 2×10^7 cells in 3 mL of RPMI 1640 medium containing 10% fetal calf serum (RPMI-10% FCS). These cells were overlayed onto 2 mL of 14.5% metrizamide gradient column. After a low-speed centrifugation (200 *g* for 10 min), cells from the interface were collected and washed twice in RPMI-10% FCS. The pellet was resuspended in another 3 mL of the same medium and the above procedure was repeated. Cells from the interface were collected and used for analysis. This preparation resulted in DC enrichment up to 85–90%, which was analyzed by FACS analysis using monoclonal antibody 33D1 (mAb 33D1 was kindly provided by Dr. Ralph Steinman, the Rockefeller University, New York, NY). Other cell types such as T cells (Thy 1.2^+), Mϕ (Mac-1^+), and B cells ($B220^+$) constituted 5–10% in these DC preparations.

Isolation of splenic Mϕ from the spleen

The isolation method used has been described previously [21]. Briefly, the splenic cells were allowed to adhere onto a plastic tissue culture T-150 flask for 90 min at 37°C. The adherent cell population was scraped off and allowed to re-adhere for another 1 h at 37°C. The re-adhered population was dislodged and resuspended in RPMI 1640 with 5% fetal bovine serum. Flow cytometric analysis using mAb of F4/80 revealed that this population contained 70–80% Mϕ and <1% DC.

Transfection of DCs and Mϕ

DCs were transfected with pc-gB DNA, pc-ICP27, or vector (pc-DNAI) in the presence of DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate) as described previously [20]. Briefly, 200 µL of phosphate-buffered saline (PBS; pH 7.2), 15 µL of DOTAP, and 50 µg of DNA were mixed and allowed to stand for 10 min at room temperature. DCs (5×10^6) in 1 mL RPMI 1640 medium containing 5% FCS (heat inactivated) were then added to the DNA-DOTAP mixture and incubated for 3 h at 37°C with occasional shaking. Finally, the cells were washed three times in PBS (pH 7.2) and used for injections.

Gene expression in the DC and Mϕ

To evaluate the gB-DNA expression in the transfected APCs, a procedure described by Rouse et al. [20] was followed. Cells (10^6) of each enriched type (DC or Mϕ) that were transfected with plasmid construct were incubated for 3, 24, and 48 h at 37°C. Cells treated with vector DNA that did not contain HSV antigen or DOTAP alone served as the negative controls. Test and control group cells were harvested at the above indicated time points and washed four times in 20 volumes of RPMI-10% FCS by centrifugation at 1000 rpm for 10 min. After the fourth wash the cell pellets were dissolved in 1 mL of Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) and the total cellular RNA was extracted and treated with RNase-free DNase (Promega, Madison, WI) to remove the contaminating plasmid DNA. These samples were reverse-transcribed by use of oligo-dT(18) primers and superscript II (both from Life Technologies, Inc., Grand Island, NY). The levels of β-actin and gB transcriptions were measured with the use of a competitive quantitative polymerase chain reaction (PCR) employing a multispecific competitor as described elsewhere [22]. To minimize the variations due to efficiencies of RNA extraction and reverse transcription, the values were expressed as the ratios of gB and β-actin messages. Culture supernatants collected after the fourth wash from the 3-h samples were screened for the presence of gB-DNA (plasmid DNA) by the PCR procedure described above.

Radioimmunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) detection of protein expression

To demonstrate the expression of gB protein in transfected Mϕ and DC, enriched cell populations were transfected with pc-DNA encoding gB according to the transfection protocol described earlier [20]. The de novo synthesized proteins were labeled with 100 µCi/mL of ^{35}S-methionine (sp. act. 1190 Ci/mmol, ICN Radiochemicals, Irvine, CA, catalog no. 51001 H) in methionine-free medium (Sigma Chemicals, St. Louis, MO). DC and Mϕ transfected with control plasmid (pc-DNA) served as the negative control. Lysate of CV-1 cells infected with recombinant vaccinia virus expressing gB was used as the positive control. The cells were harvested after 24 and 48 h of transfection and were suspended in radioimmunoprecipitation assay (RIPA) buffer containing 1% NP40 and sodium deoxycholate, 1% Triton X-100 (Sigma) and 100 µM each of the protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, and leupeptin, all from Sigma). The resulting total cell lysates were then incubated with polyclonal sera raised against HSV1 in BALB/c mouse absorbed to protein-A sepharose beads (Sigma). The immunoprecipitated samples were resolved by electrophoresis in 10% SDS-polyacrylamide gels. The gel was enhanced with En^{3}Hance (New England Nuclear, Boston, MA) for 1 h before drying and autoradiography.

Immunization

BALB/c mice were immunized on days 0 and 7 with 10^6 DNA-transfected APC (DC or Mϕ) via the quadriceps muscle. The same number of mice received 90 µg of gB or ICP-27-DNA on the same days. Mice received naked pc-DNAI vector plasmid (vector DNA) or APC transfected with pc-DNAI to serve as negative controls (DC-vector or Mϕ). Positive control mice received 10^7 plaque-forming units (PFU) HSV-1 (KOS) intramuscularly on days 0 and 7. On day 14, mice were either challenged with HSV-1.17 or splenocytes were collected from the mice to study the cellular immune responses. In addition, sera were collected from the mice for antibody studies. In some experiments mice were immunized only once with nDNA or with transfected DC or Mϕ. These animals were challenged at week 5 with HSV-1.17. Before the challenge, blood was collected from these animals (from retro-orbital sinus) and the antibody pattern was studied by enzyme-linked immunosorbent assay.

Virus infections

Immunized mice were challenged with HSV-1.17 as described elsewhere [7]. Briefly, on day 14, just before challenge the left flanks of anesthetized mice were depilated and scarified and 10–20 µL containing 10^4 ($10\ ID_{50}$), 10^5 ($100\ ID_{50}$), or 5×10^5 PFU ($500\ ID_{50}$) of HSV-1 (17 strain) was added and the flanks were gently massaged. Animals were observed daily for ipsilateral zosteriform lesions, change in general

behavior, hindlimb paralysis, encephalitis, and mortality. Severity of the lesions were scored in a blinded fashion as follows: 1+, vesicle formation; 2+, local erosion and ulceration of the local lesion; 3+, mild-to-moderate ulceration; 4+, severe ulceration, hindlimb paralysis, and encephalitis. Animals with 4+ lesions were killed because animals with lesions of such severity never recover.

Ab assays

Sera collected on day 14 were used to screen for gB specific and HSV-specific immunoglobulin G using a standard quantitative enzyme-linked immunosorbent assay technique described previously [7].

Neutralization assay

The virus neutralization assay was performed as mentioned elsewhere [7]. Briefly, 50 μL of heat-inactivated serum were added in varying dilutions to flat-bottom 96-well microtiter plates (Falcon, Oxnard, CA). To this, 50 μL of 200 PFU live HSV-1 (KOS strain) was added, and the sealed plate was incubated for 18 h at 4°C. To this mixture, 50 μL of Low-Tox rabbit complement (one-quarter dilution) were added and incubated for 1 h at 37°C. The serum dilution that neutralized 50% of the virus was considered as the titer.

Lymphoproliferation assays

Details for performance of these assays have been published elsewhere [7]. Briefly, immune splenocytes (responders) were collected and restimulated in vitro (4 days at 37°C) with varying ratios of stimulator cells. Stimulator cells used were x-irradiated naive syngeneic spleen cells infected at a multiplicity of infection (MOI) of 1.5 with UV-HSV-1 (KOS strain). Responder-to-stimulatory ratios ranged from 10:1 to 0.3:1. Eighteen hours before the harvest, [^{3}H]TdR (thymidine; ICN Radiochemicals, catalog no. 2404105) at a concentration of 1 μCi/well was added to all wells. After harvesting, the radioactivity in pelleted cells was measured with a beta scintillation counter (Inotech, Lansing, MI, Trace-96 program). Results were expressed as total counts per minute (cpm) ^{3}HTd incorporation as well as by standard stimulation index [stimulation index = (cpm of responders with HSV-infected stimulators/cpm of responders with naive stimulators)].

Cytokine studies

Immune splenocytes obtained from various vaccine-recipient mice were screened for their cytokine profile by a procedure described previously [7]. For these assays, 10^6 cells were suspended in 1 mL of RPMI-10% FCS in a 12-well tissue culture plate. Cells from each vaccinated group were divided into three sets. One set of 10^6 cells were in vitro restimulated with UV-HSV-1 (KOS; MOI 1.5 before UV inactivation) and the other set with 5 μg of concanavalin A. One set remained unstimulated with any of the stimulants. All of these cells were incubated at 36°C for 72 h and the supernatant from each well was collected, aliquoted, and stored at −20°C until used. These supernatants were analyzed for various cytokines as per the previously described procedures [7]. Interferon-γ (IFN-γ) was detected by using an IFN-γ enzyme-linked immunosorbent assay minikit (Endogen, Cambridge, MA). Interleukin (IL)-2 and IL-4 were detected by bioassays using CTLL-2 and CT4.S cells (cells and IL-4 were kindly provided by Cynthia Watson, National Institutes of Health, Bethesda, MD), respectively. Concentrations of the cytokines were derived from the standard curve.

Delayed-type hypersensitivity (DTH) assays

On the day of testing, each mouse was injected with 20 μL of 10^5 UV-inactivated HSV-1.1 KOS (10^6 PFUs before inactivation) in the right ear and the same volume of vero cell extract in the left ear. The right and left ear thicknesses of each mouse were measured with a screw gauge meter (Oditest, H. C. Kroeplin GHBH, Germany) and recorded individually. The thickness was measured at 1 h before ear injection, then at 24, 48, and 72 h after injections and the values were represented as $n \times 10^{-2}$ mm. The mean ear thickness of each ear from each group of animals was calculated and the mean increase between before and 48 h after injections was compared between the various vaccine recipients.

Cytotoxic T cell (CTL) assay

Spleen cells (effector cells) collected on day 14 after immunization were measured for the presence of MHC-restricted CTLs by a procedure that has been described elsewhere [21]. Briefly, effector cells were restimulated in vitro with UV-HSV-1 (KOS; MOI = 5) in RPMI-10% FCS and incubated at 37°C for 5 days. These effectors were allowed to react with HSV-1 (KOS strain; MOI = 1.5) -infected target cells such as EMT-6 (syngeneic) or EL-4 (allogeneic). EMT-6 cells that were not infected with virus served as mock controls. A standard 4-h ^{51}Cr release assay was performed.

Statistics

Student's *t*-test was used for evaluating statistical significance.

RESULTS

Levels of immune protection induced

Groups of immunized mice were challenged intracutaneously with different doses of HSV-1.17 and animals were followed for the ensuing 14 days for signs of disease. Unimmunized mice usually develop zosteriform lesions on the flank by day 7 and die of encephalitis by day 14. As shown in **Figure 1**, this was the fate of mice immunized with vector DNA not encoding any HSV proteins. However, the majority of mice immunized with plasmid DNA encoding either gB or ICP 27 resisted challenge with 10 ID_{50} of virus. In mice immunized with DC or Mϕ that had been transfected with DNA, markedly different results were obtained. Whereas all mice immunized with transfected DC resisted the 10 ID_{50} challenge, all who received transfected Mϕ succumbed. This pattern of responsiveness held true in two repeat experiments of like design. The results with low-dose challenge indicate that transfected DC provide even greater protection than nDNA alone even though the latter mice probably received a much higher dose of DNA.

On challenge with a 100 ID_{50} of virus, the superior protective efficacy of DC delivery became even more apparent (Fig. 1). Most mice immunized with nDNA succumbed to challenge but the majority of mice immunized with transfected DC survived ($P < 0.001$). However, whereas HSV-immunized mice could survive 500 ID_{50} (or even more) of viral challenge, mice given DC gB DNA or DC ICP-27 DNA could not. The pattern of results obtained in mice immunized twice with DNA then challenged quite early after immunization on day 14, held true in another experiment in which mice were immunized intramuscularly on a single occasion but challenged much later at 5 weeks postimmunization (**Table 1**). The results presented in Table 1 indicate that transfected DC provided better protection against high-dose challenge than was induced by nDNA, but once again no protection, even against low-dose challenge, was observed in mice immunized with transfected Mϕ.

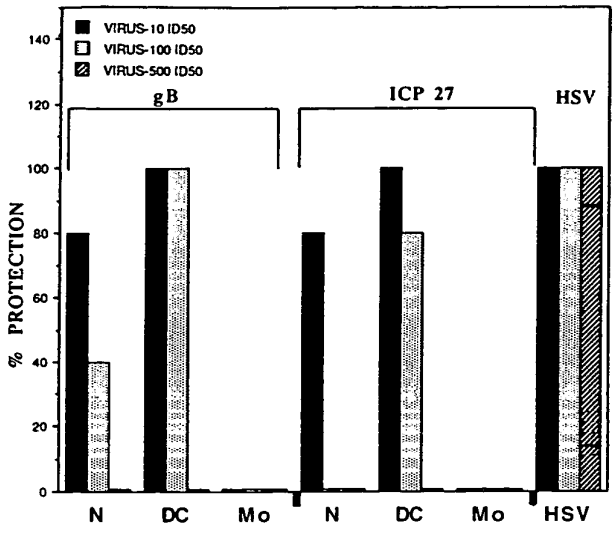

Fig. 1. Enhancement of a protection against HSV by DNA delivery via DC. Dendritic cells were isolated from BALB/c mouse spleens using a 14.5% metrizamide density gradient and macrophages by the plastic adherence method as described in Materials and Methods. Each of these enriched APC types were transfected with plasmid DNA. Either nDNA or transfected cells or HSV-1 (KOS strain) were used for immunization on days 0 and 7. N, naked DNA encoding gB or ICP-27; DC, dendritic cells transfected with gB or ICP-27 DNA; Mϕ, macrophages transfected with DNA. On the 14th day mice were challenged intracutaneously with different doses of HSV-1.17. Filled bars, the percent of mice protected on challenge with 10 ID_{50}. Stippled bars and hatched bars show the protection when challenged with 100 and 500 ID_{50} virus doses, respectively. Not shown were the groups that received vector DNA (negative control), which were all succumbed at day 14. Figures represent the protection values obtained on day 14 post-challenge. Animals that did not develop any lesions were considered protected animals.

Plasmid DNA in transfected DC and Mϕ is expressed

Our observation that immunization with transfected DC induced protection, whereas transfected Mϕ failed to do so requires explanation. To exclude the simple explanation that transfection was only successful in the DC population, highly enriched populations of both DC and Mϕ were transfected with pc-DNA encoding gB and washed cells were held in culture for various times before harvesting their RNA. The RNA was subjected to qualitative and quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) to detect and compare expression of mRNA for gB. Aliquots of cells immediately after transfection and washing were also used to test their immunogenicity. Again the same pattern of results was obtained with protection occurring with DC but not with Mϕ (data not shown). As regards mRNA, both transfected DC and Mϕ cell populations expressed gB mRNA. This was detectable at all three time points measured (3, 24, and 48 h; **Fig. 2**). Attempts to detect protein levels by radioimmunoprecipitation assay indicated that both cell types produced gB protein (**Fig. 3**). Interestingly, cell lysates of transfected macrophages but not DC additionally showed a degraded form of gB. We can conclude from our results that the failure of transfected Mϕ to induce protection was not because such cells failed to express the plasmid DNA.

TABLE 1. Immunization of Mice with a Single Dose of NDNA or Transfected APC[a]

Groups	Days	Virus challenge dose (ID_{50}) 10	100	500
gB-DNA	7	0/5	3/5	4/5
	14	0/5	5/5 (D)[b]	5/5 (D)
DC-gB	7	0/5	0/5	3/5
	14	0/5	0/5	5/5 (D)
ICP-27 DNA	7	0/5	4/5	5/5
	14	0/5	5/5 (D)	5/5 (D)
DC-ICP27	7	0/5	1/5	4/5
	14	0/5	1/5 (1=D)	5/5 (D)
HSV	7	0/5	0/5	0/5
	14	0/5	0/5	0/5

BALB/c mice were immunized on day 0 with nDNA, APC, or HSV as described in Materials and Methods. Animals were challenged on week 5 with different doses of HSV-1.17 such as 10, 100, or 500 ID_{50} (10 ID_{50} = 1 x 10^5 PFU/mouse) and observed for 14 days. The number of animals that developed lesions out of the number exposed is shown.

[a] The groups that were dead by day 14 post-challenge are not shown. Those groups were the mice that received vector DNA directly or via APC; Mϕ-gB; Mϕ-ICP-27.

[b] D, lesion-developed mice that died.

Measurement of immune responsiveness

In an attempt to provide a mechanistic explanation for the enhanced levels of protection observed following immunization with DC-incorporated nDNA, various measures of

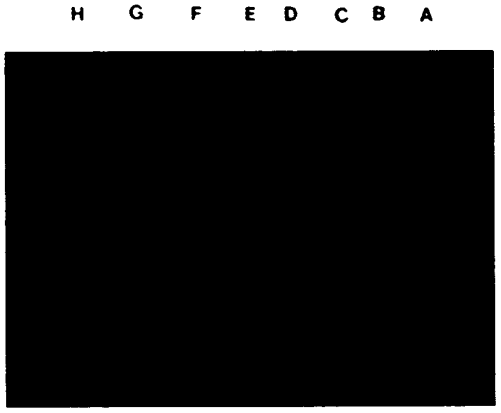

Fig. 2. Qualitative profile of housekeeping gene (HPRT) and gB expression. Total RNA derived from equal numbers of cells was treated with RNase-free DNase and was reverse transcribed using subscript II and Oligo dT(18) primers. The resultant cDNA were taken up in equal volumes and amplified using HPRT and gB specific primers. The PCR products were analyzed on a 2% agarose gel, stained with ethidium bromide, and photographed. Also the supernatants from the fourth wash were examined for the presence of free pc-DNA gB by PCR. Lanes A, DNA marker (1.0-kb ladder); lane B, DC-gBDNA, 3 h; lane C, DcgBDNA, 24 h; lane D, DcgBDNA, 48 h; lane E, DC-gB DNA, supernatant; lane F, Mϕ-gBDNA, 3 h; lane G, Mϕ-gBDNA 24 h; lane H, Mϕ gBDNA, 48 h.

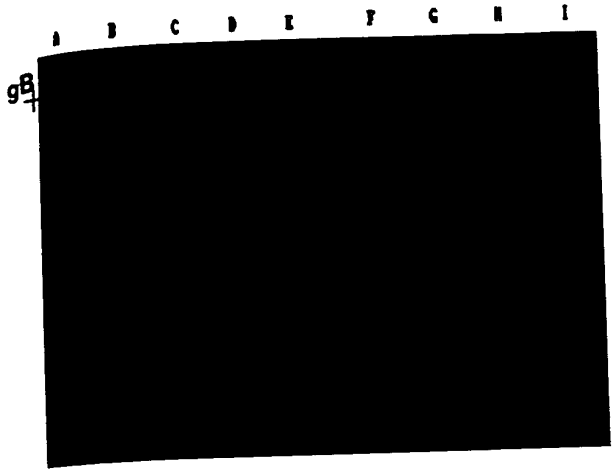

Fig. 3. gB-protein detection by radioimmunoprecipitation. Transfected DC or Mϕ were grown in methionine-free medium supplemented with L-[^{35}S]-methionine for 24 and 48 h. At these time points, cells were harvested and lysed in RIPA buffer (see Materials and Methods). Lysates were then treated with mouse anti-HSV serum adsorbed onto Protein-A-Sepharose beads. The immunoprecipitates were resolved on a 10% SDS-polyacrylamide gel, enhanced, dried, and autoradiographed. gB protein (~110 kDa) is shown at lanes B, C, F, and H. Also a degraded form of the same protein could be seen in lane F. Lane A, DC-vector DNA, 24 h; lane B, DcnDNAgB, 24 h; lane C, DC-nDNA-gB, 48 h; lane D, DC-vector DNA, 48 h; lane E, Mϕ-vector DNA, 24 h; lane F, Mϕ-nDNA-gB, 24 h; lane G, Mϕ-vector DNA, 48 h; lane H, Mϕ-nDNA-gB, 48 h; lane I, non-transfected DC/Mϕ cell lysate.

humoral and cellular immunity were made. Most antibody measurements were made on day 14 following DNA immunization, at which time levels are low and still rising. In such samples no significant differences in levels of Ab to gB detectable by either ELISA or neutralization were observed between nDNA and DC-DNA immunized mice (data not shown). In none of the mice immunized with Mϕ-DNA was serum antibody to HSV detectable. In contrast, in serum pools from animals immunized on a single occasion and examined at 5 weeks, antibody responses were readily detectable by ELISA and neutralization in gB DNA-immunized mice (**Table 2**). Moreover, the ELISA titer in animals immunized with DC-DNA was more than fivefold higher and showed a higher IgG_{2a}-IgG_1 ratio than the pool from animals immunized with gB nDNA.

Measurement of cell-mediated immunity also revealed some differences between DC-DNA-immunized and nDNA-immunized mice. Most striking were the differences in antigen-specific proliferative responses (**Table 3**). Proliferative responses in spleen cells from mice immunized with both DC-gB DNA and DC ICP-27 DNA were both significantly higher than were those observed in mice immunized with nDNA ($P = 0.003$). The proliferative responses observed in splenocytes from mice immunized with transfected DC were similar to those observed in HSV-immunized mice. The data also indicate that mice immunized with transfected Mϕ failed to respond.

Antigen-stimulated cultures were also compared for their pattern of cytokine responsiveness measuring levels of IFN-γ, IL-2, and IL-4 production. Splenocytes obtained from nDNA-gB- or ICP-27-vaccinated mice produced higher levels of Th1 cytokines on in vitro restimulation with HSV (**Table 4**). A similar cytokine profile was also observed on DC-DNA vaccination with no apparent increase in the response. However, mice immunized with Mϕ gB DNA failed to show cytokine induction, once again indicating their failure to respond to the immunization.

On analysis of DTH reactions to HSV antigen, DC gB DNA- and DC ICP-27 DNA-immunized mice were observed to have enhanced responses over those occurring in mice immunized with nDNA (**Table 5**). This may indicate a more vigorous $CD4^+$ T cell response in mice immunized with transfected DC.

Finally, levels of CTL induction were compared in the various groups of mice. For these analyses, pooled splenocytes from each group were stimulated in vitro with HSV antigen for 5 days followed by measurement of CTL activity. CTL activity was noticed in the groups that received nDNA-ICP-27 or DC-DNA-ICP-27. However, no statistical differences in responsiveness could be found (data not shown).

DISCUSSION

In this study we demonstrate that superior immunogenicity results from introducing naked DNA vaccines into dendritic cells ex vivo and using such cells for immunization. This was shown by comparing the level of protection against HSV challenge induced by naked DNA encoding HSV proteins given directly with the same source of DNA transfected in vitro into DC. Whereas both immunization protocols protected against minimal viral challenge, the superiority of the DC delivery approach became evident as viral challenge levels were increased. Our results add to the increasing evidence that DC act as potent carriers of immunogens. Most previous reports have involved tumor immunity and have used DC to deliver proteins, peptides or even RNA [12–

TABLE 2. Enhancement of Antibody Production on Single Vaccination[a] of Mice with DNA-Transfected DC

	Nt. Ab[b] titer	gB-Specific antibodies (ng/mL)			
		Total IgG	IgG_{2a}	IgG_1	IgG_{2a}:IgG_1 ratio
gB-DNA	16	1,664	1,919	504	4.3
vector-DNA	<2	<100	<1	<1	–
DC-gB	32	10,240	12,757	610	20.6
DC-vector	<2	<100	<1	<1	–
Mϕ-gB	<2	<100	<1	<1	–
Mϕ-vector	<2	<100	<1	<1	–
HSV	32	23,200	28,500	2,200	13.0

[a] BALB/c mice were immunized once with nDNA or transfected APC or HSV (KOS) in the quadriceps muscle. Five weeks later, serum from these animals was collected and checked for the presence of gB-specific antibodies by ELISA.

[b] Some sera were tested for their neutralizing antibody titers by complement-assisted virus neutralization (Nt.) assay as described in Materials and Methods. The table represents the Nt. antibody titers of each sample tested. The serum dilution that caused 50% reduction of virus plaques was considered as the titer.

TABLE 3. HSV-Specific Lymphoproliferation of the Spleen Cells from DNA-Immunized BALB/c mice

Responders	HSV stimulators 10:1	HSV stimulators 0.3:1	Naive stimulators, 10:1	No stimulators	Proliferation Index
gB-DNA	532.8 ± 93.0	122.5 ± 59.3	34.0 ± 11	32.3 ± 4.0	15.9
ICP27-DNA	563.0 ± 100.0	79.0 ± 17.7	51.0 ± 6.9	75.3 ± 48.0	11.0
vector-DNA	45.8 ± 18.0	32.8 ± 11.8	30.3 ± 10	26.5 ± 8.5	1.5
DC-gB	5740.5 ± 126.6	100.0 ± 64.6	24.5 ± 8.8	33.7 ± 12.6	234.3
DC-ICP27	5191.0 ± 860.4	43.8 ± 4.8	35.3 ± 8.4	32.3 ± 12.6	147.0
DC-vector	58.2 ± 38.1	49.5 ± 28.8	24.0 ± 8.8	21.3 ± 7.1	2.4
Mϕ-gB	201.6 ± 82.7	37.5 ± 17.7	39.4 ± 1.4	28.5 ± 5.0	5.2
Mϕ-ICP27	151.5 ± 37.5	55.5 ± 24.8	34.5 ± 2.1	19.0 ± 12.7	4.4
Mϕ-vector	105.0 ± 35.3	19.0 ± 5.7	29.0 ± 1.4	15.0 ± 7.1	3.6
HSV	7561.0 ± 1499.0	105.5 ± 97.3	42.8 ± 8.6	44.3 ± 18.1	176.7

BALB/c mice were immunized with nDNA or DNA-transfected APCs on days 0 and 7. Mice vaccinated with HSV-1.KOS served as the positive immune group. On day 14 immune splenocytes recovered were in vitro restimulated with UV-HSV-1 (KOS) -infected stimulator cells (syngeneic splenocytes). The table represents the stimulation index (SI) obtained at a responder-to-stimulator ratio of 10:1. Stimulation index, CPM with HSV infected stimulators/CPM with naive stimulators. Responder + stimulators were incubated for 5 days at 37°C and harvested. Eighteen hours before the harvest, 1 μCi/well of [^{3}H]thymidine was added. Harvested cells were measured for the radio activity using Inotech beta ray counter. As a positive control, each responder population was stimulated with concanavalin A (5 μg/mL). [^{3}H]thymidine was added to this group at 48 h and harvested at 72 h. All the groups showed a mean CPM of 25,000 to 30,000.

18]. Our report may be the first to use DC to deliver nDNA and to focus on protection against an infectious disease.

The surprising observation in 1992 that naked plasmid DNA induced effective immunity following parenteral or mucosal administration was soon confirmed in many systems [1–3]. We, for example, showed the value of nDNA to protect against HSV infection [7, 8] and these reports were later confirmed by others [23, 24]. Indeed, commercial groups are seriously considering the use of nDNA to vaccinate against HSV as well as many other agents. However, some significant obstacles still remain before nDNA can be considered as the panacea for vaccinology. One important issue is efficacy. In our own studies on immunity to HSV in mice, immunization with nDNA intramuscularly [7, 8] or even intranasally [25], was shown effective but less so than the alternative more traditional approaches. Accordingly, mice immunized with attenuated virus or recombinant vaccinia viruses expressing HSV proteins, resisted far higher doses of viral challenge than did nDNA-immunized mice [9]. This observation held true for studies with DNA encoding at least two HSV proteins: gB, a major glycoprotein and ICP-27 a regulatory protein [7, 8]. Our present observation that delivery via DC enhanced immunity, although perhaps not a practical vaccination procedure against an infectious disease, does support the idea that nDNA should be capable of inducing effective levels of immunity. Although DC can be given repeatedly without apparent problems even to humans [15], for practical purposes it may be necessary to optimize the delivery to DC in vivo or to exploit strategies that attract and activate such cells. Regarding the latter idea, Ertl's group has shown the value of co-administration of DNAs for granulocyte-macrophage colony-stimulating factor (GM-CSF) with a virus glycoprotein DNA to enhance immunogenicity [26]. We have observed similar effects with nDNA for HSV proteins given along with GM-CSF DNA to enhance anti-HSV immunity [25]. Thus far, whether or not GM-CSF induced

TABLE 4. HSV-Specific Cytokine Production of the Spleen Cells from Various Vaccine Recipient Mice

Immunogen	IFN-γ (pg/mL)	IL-2 (pg/mL)	IL-4 (pg/mL)
pc-gB	2,400	2,150	<10
pc-ICP-27	1,800	2,200	<10
pc-vector	<50	<50	<10
DC-gB	2,600	2,500	<10
DC-ICP27	2,300	2,400	<10
DC-vector	<50	50	<10
Mϕ-gB	<50	<50	<10
Mϕ-vector	<50	<50	<10
Mϕ-ICP-27	<50	<50	<10
HSV	2,250	2,500	50

BALB/c mice were immunized with nDNA or transfected dendritic cells or HSV-1 (KOS strain) on days 0 and 7. Splenocytes collected on day 14 were in vitro-restimulated with UV-inactivated HSV-1 (KOS) for 72 h. Culture supernatants collected at 72 h were screened for cytokines IFN-γ (ELISA), IL-2 (CTLL-2 bioassay), and IL-4 (CT4.S bioassay). Unstimulated culture supernatants had no detectable levels of screened cytokines. Similar results were obtained from three experiments and the table represents one of the representative experiments.

TABLE 5. Analysis of the DTH of the Vaccinated BALB/c Mice

Immunogen	Left ear (vero cell extract)	Right ear (UV HSV-1.17)
gB-DNA	2.0 ± 1.2	7.8 ± 1.2
ICP-27-DNA	1.8 ± 0.5	7.3 ± 1.3
vector-DNA	2.8 ± 0.5	2.3 ± 0.9
DC-gB	2.8 ± 0.5	15.0 ± 1.4
DC-ICP-27	2.5 ± 0.6	12.0 ± 1.6
DC-vector	2.0 ± 0.8	1.8 ± 1.0
HSV-1 (KOS)	2.3 ± 1.0	16.0 ± 4.0

BALB/c mice immunized with immunogens were used for DTH assay on day 14. Test antigens injected were 20 μL of HSV-1-17 (10^5 PFU before UV inactivation) or vero cell extract. Ear thickness was measured before the injection and 24, 48, and 72 h after the ear injection. Values are mean ± SD increase in thickness at 48 h after ear challenge. Values are represented as $n \times 10^{-2}$ mm. Mice received Mϕ transfected with gB or ICP-27 or vector DNA did not show a mean increase of more than 3.0 mm × 10^{-2} mm.

enhancement functions occurs solely by effects on DC activity in vivo needs formal demonstration. Interestingly, of the many cytokines shown to act as immune enhancers against certain tumors, GM-CSF is often found to be the most effective [27].

Although our studies clearly showed improved resistance following DC delivery, it seems likely that further improvements may be attained perhaps reaching to the level of immunity achieved by attenuated virus immunization. In our studies, splenic DC were used, not DC from bone marrow, which in some tumor systems appears to be superior [28]. It also seems likely that the co-administration of activating or costimulatory cytokines may enhance the efficacy of DC delivery. This issue is currently under investigation in our laboratory. Curiously, whereas DNA-transfected DC achieved immunogenicity, the use of similarly transfected Mϕ did not. Reasons for the disparities between DC and Mϕ were not identified but the observation that DC are superior APC for primary immune responses both in vivo and in vitro has become widely recognized [29]. We considered that the failure of transfected Mϕ to provide immunity was because such cells failed to adequately take up and express the DNA. As judged by RT-PCR, looking for RNA expression in transfected cells, this proved not to be the case. Indeed the Mϕ-transfected population expressed RNA as well as protein about equally as well as did the DC population. It remains a possibility that the failure of Mϕ to impart immunogenicity was associated with their lower content of MHC Class II proteins and essential costimulators [11]. Conceivably, Mϕ might also process peptides less effectively than do DC. Some ideas about the superior antigen-presenting function of DC over other cell types for protein immunogenicity recently received an excellent review [33].

Measurements of levels of immune responses occurring in nDNA compared with DC DNA-immunized animals were done in an attempt to identify mechanisms responsible for increased resistance of the DC DNA-immunized animals. Some clues emerged. Accordingly, elevated responses were evident primarily in $CD4^+$ T cell function, presumably that subset with a type 1 cytokine-producing profile. Elevated DTH reactions, a correlate of Th1 responses against HSV [30], and IgG_{2a} antibody levels were noted. T cell proliferation responses to in vitro stimulation with HSV were also markedly elevated in DC-DNA-immunized mice, but efforts to detect increased amounts of type 1 cytokines in lymphoid cells from DC-DNA-immunized mice were not successful. Interestingly, significant effects on CTL levels were not detected, a surprising observation because in several examples where delivery of proteins and peptides within DC have led to enhanced tumor immunity, the effect was linked to a better CTL response [29, 31]. It remains possible that our assays were insufficiently sensitive to detect differences because in the HSV system responses are modest and only demonstrable following secondary in vitro stimulation [32]. Analysis by limiting dilution to enumerate CTL-p may be required to reveal changes in the $CD8^+$ T cell response. Both this approach as well assays to enumerate individual cytokine-producing cells are under investigation to guide the further improvement of nDNA immunization approaches.

ACKNOWLEDGMENTS

This work was supported by Grant AI14981.

We appreciate the technical help of William S. Wire and the word processing wizardry of Paula Keaton.

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